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Supporting Information

Activity-Based Profiling of Retaining β-Glucosidases: A Comparative Study

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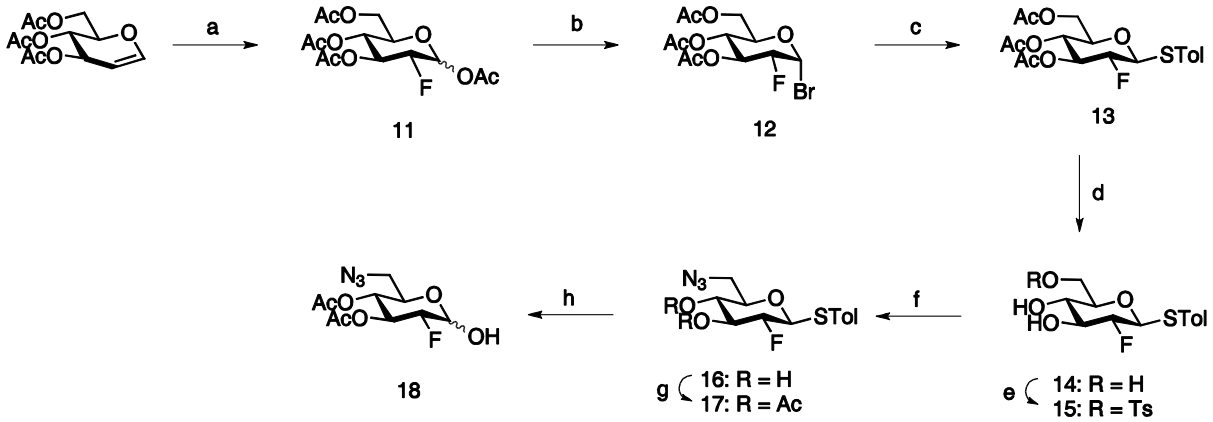
SUPPORTING INFORMATION

Synthesis of the probes

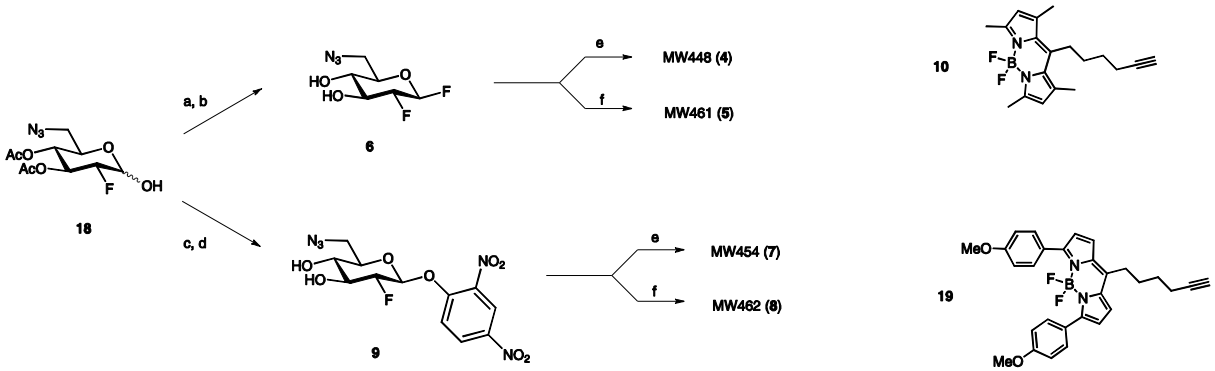
The synthesis of 2-fluoro-glucopyranose probes commences with the synthesis of 2-fluoro-glucopyranoside **11**. To obtain this intermediate we applied the direct electrophilic fluorination of 3,4,6-tri-*O*-acetyl-D-glucal method using Selectfluor, as developed by Dax et al.[1] The major drawback of this facile method is that an epimeric mixture is produced of *manno*/*gluco*-pyranosides in a almost equal ratio. However, upon acetylation of the anomeric hydroxyl, the *gluco* epimer can be isolated.



Scheme 1. Synthesis of key intermediate **18**. Reagents and conditions: a) Selectfluor, NO_2Me, then Ac_2O, pyridine; b) HBr/AcOH, DCM; c) TolSH, TBAB, aq. KOH, $CHCl_3$ (83% over 2 steps); d) NaOMe, MeOH (quant.); e) Ts_2O, NEt_3, dioxane (52%); f) NaN_3, DMF, 80 °C; g) Ac_2O, pyridine (69% over 2 steps); h) NBS, acetone/H_2O (86%).

Starting from 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranoside **11**, the anomeric acetyl was brominated using HBr/AcOH in DCM, and after aqueous work-up subsequently substituted with an *S*-toluyl moiety to yield β-thio compound **13** as the sole anomer (83% over two steps). Deacetylation using Zemplén conditions resulted in triol **14** in quantitative yield. When compound **14** was treated with tosyl chloride in pyridine to regioselectively introduce a tosyl functionality at C-6, a substantial amount of the 6-chloride was isolated together with the desired 6-*O*-tosylate. Since the 6-chloride showed a similar behavior on TLC as the 6-*O*-tosylate, it was impossible to efficiently isolate

desired compound **15**. However, formation of the 6-chloride was suppressed when compound **14** was reacted with tosyl anhydride in dioxane and compound **15** was obtained in moderate yield (52%). Subsequent substitution of the tosyl functionality with NaN_3 in DMF at 80 °C, followed by acetylation of C-3 and C-4 (Ac_2O/pyridine) gave compound **17** in 69% over two steps. In a first attempt to synthesize β-fluoride compound **6**, thioglucoside **17** was treated with DAST/NBS in DCM over 3 days to produce solely the α-fused anomeric fluoride product in 85%. A possible explanation for this high α-selectivity is that the activated β-thio functionality is not very prone to leave and is therefore substituted in an S_N2-like manner to produce the α-product. The electron-withdrawing fluoride at C-2 and the azide at C-6 are believed to lie at the base of this inreactivity. We now turned our attention to the hemiacetal **18**, from which both the anomeric fluoride and the 2,4-dinitrophenyl glucosides could be synthesized. To obtain key intermediate **18**, the anomeric thio functionality was hydrolyzed using the NIS/TFA method.[2] Surprisingly, the reaction was very slow and resulted after long reaction times and an excess of reagents in a mixture of the desired hemiacetal product and a diastereomeric mixture of β-sulfoxides. Switching of the solvent system from DCM to acetone/H_2O resulted in practically no conversion of starting compound **17**. The use of NBS as the electrophilic thio activator gave the best results, since TLC analysis revealed quick consumption of the starting compound (~ 10 min) to the sulfoxides, which were hydrolysed with additional NBS overnight to yield hemiacetal product **18** in 86%. When hemiacetal **18** was treated with DAST at -45 °C for 3 h, a mixture of anomeric fluorides was obtained with the β-fused product as the major isomer (α : β = 1 : 4). Fast deacetylation using stoichiometric NaOMe resulted in the formation of a substantial amount of the α-*O*-methyl glucoside by direct substitution of the anomeric fluoride functionality. On the other hand, a catalytic amount of NaOMe in MeOH yielded MW447 **6** quantitatively. To produce 2,4-dinitrophenyl glucoside **9**, hemiacetal **18** was treated with 2,4-dinitrofluorobenzene and DABCO in DMF. A mixture of anomers was produced of which the β-fused product could be isolated in 36%. Deacetylation was accomplished under acidic conditions (AcCl in MeOH) to yield **9** in 89%.



Scheme 2. Synthesis of the 2-fluoro glucoside probes. Reagents and conditions: a) DAST, DCM (64%); b) NaOMe, MeOH (quant.); c) 2,4-dinitrofluorobenzene, DABCO, DMF (36%); d) AcCl, MeOH (89%); e) BODIPY-alkyne **10**, sodium ascorbate, $CuSO_4$, DMF (MW448 **4**: 56%, MW454 **7**: 36%); f) BODIPY-alkyne **19**, sodium ascorbate, $CuSO_4$, DMF (MW461 **5**: 21%, MW462 **8**: 32%).

Using the copper-catalyzed click reaction,[3] 6-azido-2-fluoro glucoside probes MW447 **6** and MW436 **9** were conjugated with BODIPY-alkyne **10** (green emission) and BODIPY-alkyne **19** (red emission). In this way, the four direct probes MW448 **4**, MW454 **5**, MW461 **7** and MW462 **8** were obtained (Scheme 2).

Determination of the binding-constants.

The binding-constants of the probes for almond β-glucosidase and for glucocerebrosidase were determined using an indirect fluorescent substrate assay. By plotting the residual activity versus the time the apparent rate constant could be determined. The resulting progress curves can be seen in Figure 1 and 2 (left panels). To determine the constant for initial binding (K_i) and the rate constant (k_i), the apparent rate constants were plotted versus the concentration (Figure 1 and 2 right panels).

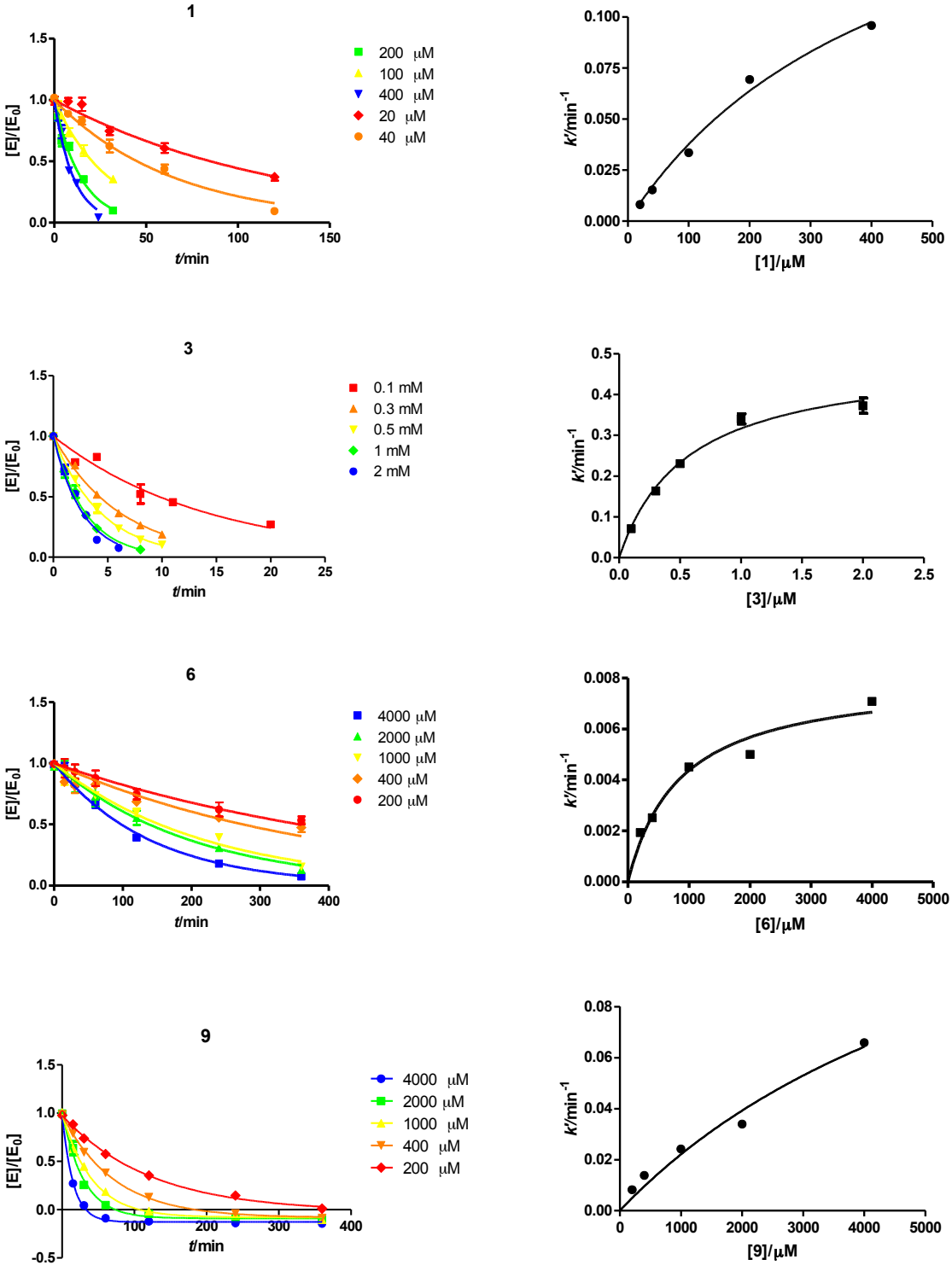


Figure 1. Determination of the binding-constants of the probes for almond β-glucosidase. (left) Progress curves. $[E]/[E_0]$ is the residual activity (fluorescence at time point x divided by the fluorescence at time point zero). (right) Kitz-Wilson plot of the pseudo-first order rate constants (*k'*).

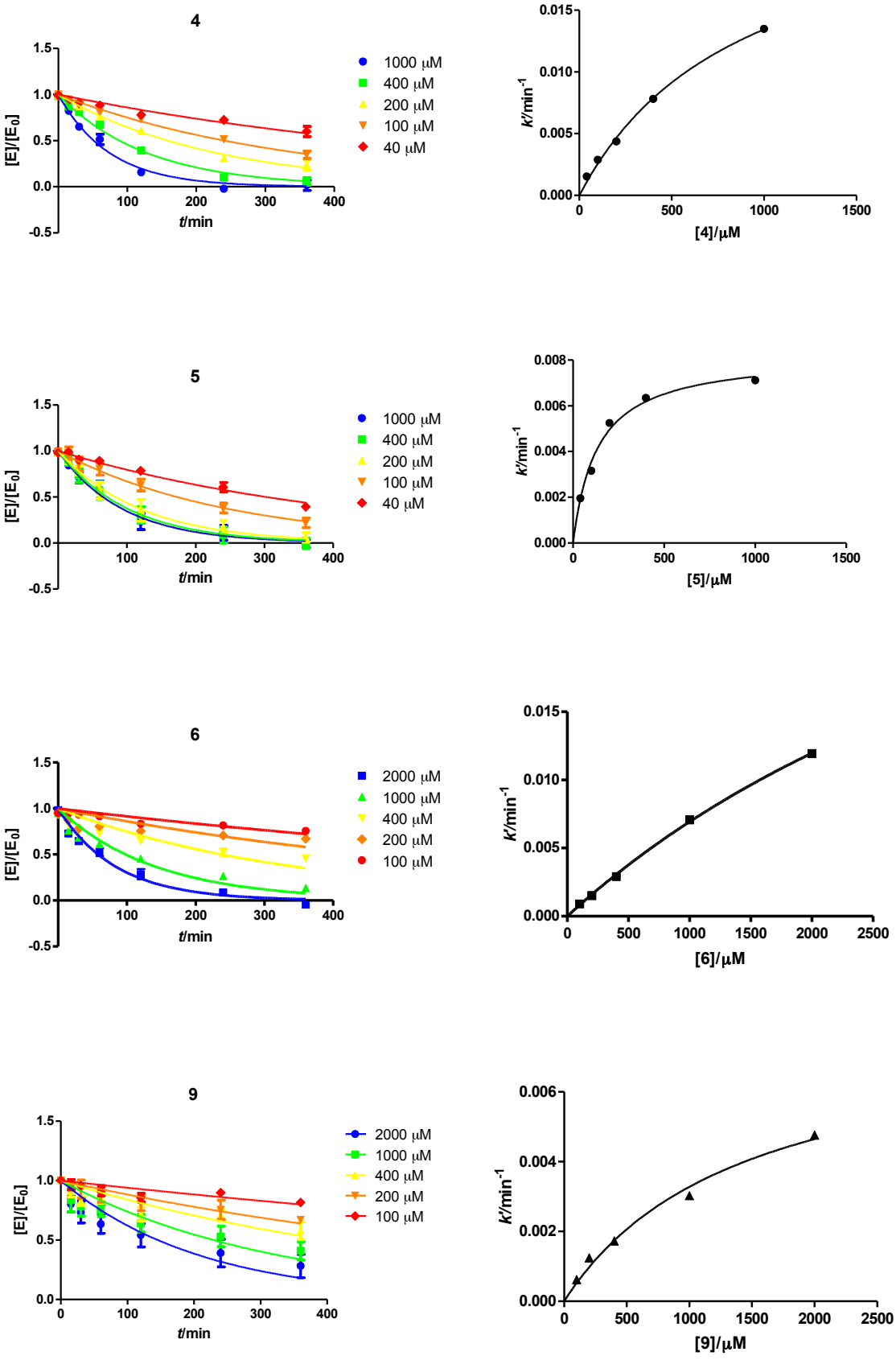


Figure 2. Determination of the binding-constants of the probes for GBA. (left) Progress curves.

[E]/[E_0] is the residual activity (fluorescence at time point x divided by the fluorescence at time point zero). (right) Kitz-Wilson plot of the pseudo-first order rate constants (*k'*).

Two-step labeling: Staudinger ligation versus copper catalyzed click-reaction.

To compare the efficiency of the two-step labeling, GBA-1 was inhibited with the probe (10 μM) and subsequently either subjected to either biotinylated Staudinger-Bertozzi phosphane **20** (400 μM for 4h in the presence of 8 M urea) or biotin-derived alkyne **21** in the presence of Cu(I) (100 μM for 16h in NaOAc buffer containing 1% SDS) (figure 3a).[4, 5] The labeled proteins were visualized by western blotting. As can bee seen in figure 3 the copper-catalyzed click ligation gave a stronger signal then the Staudinger-Bertozzi ligation. Exposure of the film for 10 seconds gave a good signal for the copper-catalyzed click reaction (Figure 3b). This is not the case for protein labeled with the two-step probe and visualized with the Staudinger ligation. These only revealed a signal after 5 minutes of exposure (3c).

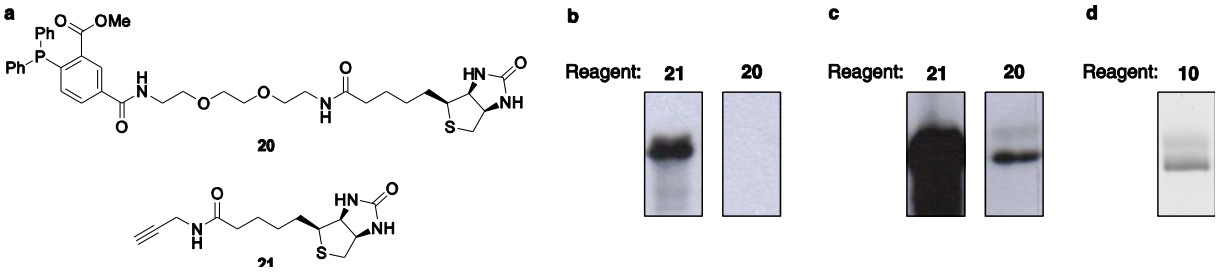


Figure 3. Functionalisation of the azido group using either Staudinger ligation or the copper catalyzed click-reaction. (a) Structures of the biotinylated compounds used. (b) 10 seconds exposure of the film. (c) 5 minute exposure of the exact same western blot. (d) Functionalisation of the azido group with BODIPY-alkyne **10**.

Labeling of retaining beta-glucosidases.

Next to labeling with the green fluorescent probes, we also labeled GBA-1 and almond β-glucosidase with probes containing a red fluorophore. Labeling with these probes is very similar to labeling with green fluorescent probes **1**, **4** and **7**. Cyclophellitol probe **2** and 2-deoxy-2-fluoroglucoside probe **5** label GBA-1 in a concentration dependent fashion, whereas dinitrophenyl containing probe **8** did not

label GBA-1 at all, a feat which was also observed for the green probes. Moreover, the red probes hardly label almond β-glucosidase (Figure 4).

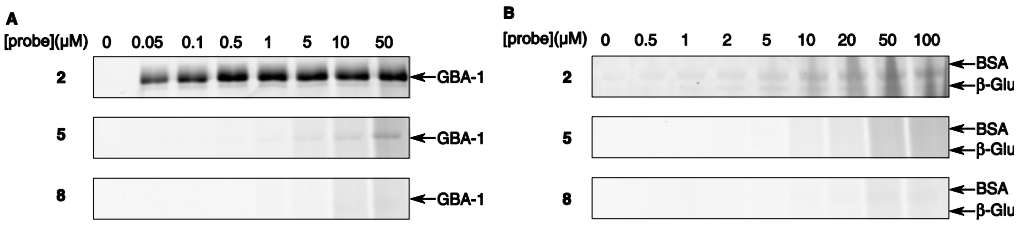


Figure 4. Labeling of beta glucosidases with red fluorescent probes **2**, **5** and **8**. (a) Labeling of almond β-glucosidase. (b) Labeling of GBA-1.

The gels of GBA-1 that was labeled the 2-deoxy-2-fluoride probes **4** and **5** revealed only a faint signal. We reasoned that this was caused by incomplete inactivation of the enzyme. The progress curves of the kinetic experiments (Figure 1a) show that only a small portion of GBA-1 is inhibited at the time and concentration used. Furthermore, the progress curves demonstrate that nearly complete inhibition of the enzyme can be achieved by treating the enzyme for 6 hours with 100-400 μM. We therefore reasoned that increasing the labeling time and the concentration would lead to an enhanced signal and indeed this is the case as can be seen in Figure 5.

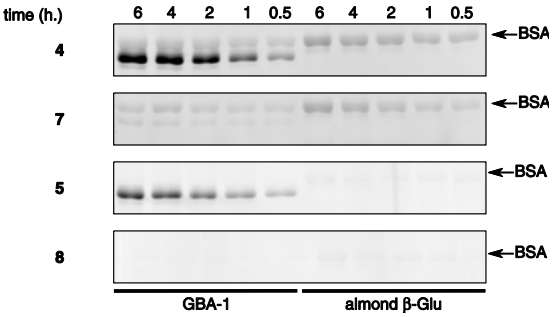


Figure 5. Time dependency of labeling with **4**, **5**, **7** and **9**.

Having optimized the labeling conditions, we tested the requirement of active enzyme for labeling as described in the main text of the manuscript (Figure 6).

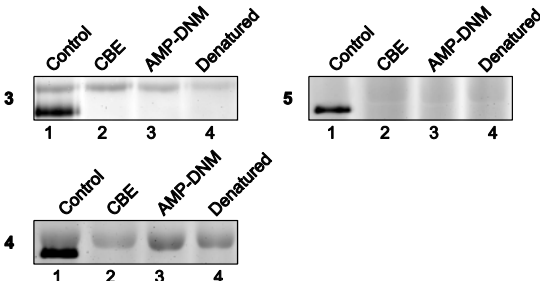


Figure 6. Labeling by **3**, **4** and **5** requires active enzyme. Lane 1: GBA-1 was treated with probe (**3**: 10 μM, **4**: 400 μM and **5**: 100 μM) at 37°C for 30 min (**3**) or 6 h (**4** and **5**). Lane 2: GBA-1 was pretreated with CBE (2 mM) for 30 min followed by incubating with probe (similar concentrations were used as in the control). Lane 3: GBA-1 was incubated with the probe in the presence of AMP-DNM (2 mM). Lane 4: GBA-1 was heat denatured with 1% SDS prior to labeling with the probe.

Finally, we tested the sensitivity of the probes. Decreasing amounts of enzyme were incubated with the probe. As shown in the manuscript the sensitivity of the two-step probes and the direct, green fluorescent probes are in the same range. The same counts for the red-fluorescent probes, except for almond β-glucosidase which is labeled far better by two-step probe **3** than the direct probe (Figure 7A). Furthermore, we tested the sensitivity of 2-deoxy-2-fluoroglycoside probes **4** and **5** for recombinant purified glucocerebrosidase. These probes are as sensitive as the cyclophellitol probes when enzyme was subjected to concentrations > 100 μM for 6h, conditions clearly not suitable for in cell-labeling.

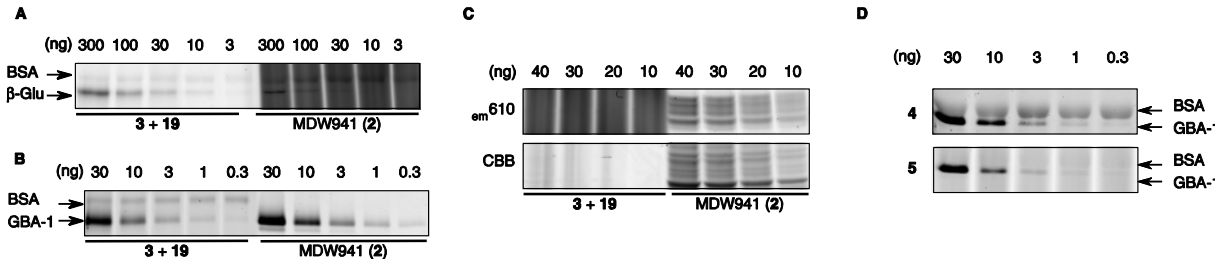
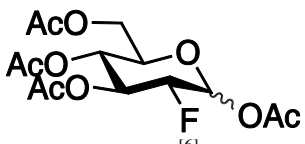


Figure 7. A, B, C) Sensitivity of the red fluorescent probe versus the two-step probe in combination with BODIPY **19**. D) Sensitivity of 2-deoxy-2-fluoroglycoside probes **4** and **5**.

Experimental section

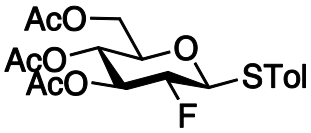
All reagents were of commercial grade and used as received unless stated otherwise. Diethyl ether (Et_2O), ethyl acetate (EtOAc), light petroleum ether and toluene were obtained from Riedel-de Haën. Acetonitrile, dichloromethane, *N*,*N*-dimethylformamide (DMF), methanol (MeOH), pyridine, tetrahydrofuran (THF) were purchased from Biosolve. Dichloromethane was distilled from CaH_2 and THF was distilled over $LiAlH_4$ prior to use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality.

Reactions were monitored by TLC analysis using Merck aluminum sheets precoated with silica gel 60 with detection by UV-absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150°C or by spraying with 20% sulfuric in ethanol followed by charring at ~150°C. Column chromatography was performed using either Baker- or Screening Device silica gel in the indicated solvents. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DMX-400 (400/100 MHz) or a Bruker AV-400 (400/100 MHz) spectrometer in the given solvent. Chemical shifts are reported as δ-values in ppm relative to the chloroform residual solvent peak or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. Spin multiplicities are given as s, d, dd, ddd, dddd, dt, t, td, q and m. High resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). LC/MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 nm and 254 nm) equipped with an analytical Alltima C18 column (Alltech, 4.6 mmD × 50 mmL, 3μ particle size) in combination with buffers A: H_2O, B: acetonitrile and C: 1% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). FT-IR-spectra were recorded on a Paragon-PE 1000.



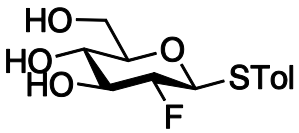
1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-fluoro-α/β-D-glucopyranoside (11). The title compound was synthesized according to a procedure described by Priebe *et al*.[6] and the analytical data is in accordance to those described. TLC: R_f 0.61 (PE/EtOAc, 1/1, v/v); IR (neat, cm^{-1}): 1036, 1211, 1369, 1747; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 6.28 (d, 0.78H, *J* = 3.9 Hz, H-1α), 5.70 (dd, 1H, *J* = 3.1, 8.1 Hz, H-1β), 5.40 (dt, 0.78H, *J* = 9.6,

12.2 Hz, H-3α), 5.29 (dt, 1H, *J* = 9.3, 14.3 Hz, H-3β), 4.95 (t, 0.78H, *J* = 9.9 Hz, H-4α), 4.92 (t, 1H, *J* = 4.9 Hz, H-4β), 4.55 (ddd, 0.78H, *J* = 4.0, 9.6, 48.5 Hz, H-2α), 4.31 (dt, 1H, *J* = 8.6, 50.9 Hz, H-2β), 4.17 (t, 0.78H, *J* = 4.7 Hz, H-6α), 4.14 (t, 1H, *J* = 4.7 Hz, H-6β), 3.88-4.00 (m, 2.56H, H-5α, H-6α, H-6β), 3.79 (ddd, 1H, *J* = 2.1, 4.4, 10.1 Hz, H-5β), 2.06 (s, 2.31H, CH_3 Ac-α), 2.03 (s, 3H, CH_3 Ac-β), 1.94 (s, 6H, CH_3 Ac-β), 1.93 (s, 4.92H, CH_3 Ac-α), 1.90 (s, 2.31H, CH_3 Ac-α), 1.89 (s, 3H, CH_3 Ac-β); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 170.1, 169.7, 169.4, 169.1, 169.1, 168.4, 168.3 (C=O Ac), 90.8 (d, *J* = 24 Hz, C-1β), 87.9 (d, *J* = 190 Hz, C-2β), 87.9 (d, *J* = 22 Hz, C-1α), 85.8 (d, *J* = 193 Hz, C-2α), 72.2 (d, *J* = 19 Hz, C-3β), 72.2 (C-5β), 70.2 (d, *J* = 19 Hz, C-3α), 69.1 (C-5α), 67.2 (d, *J* = 7 Hz, C-4β), 67.0 (d, *J* = 7 Hz, C-4α), 61.0 (C-6), 20.4, 20.2, 20.1 (CH_3 Ac); HRMS: $[M+Na]^+$ calcd for $C_{14}H_{19}FO_9Na$ 373.0905, found 373.0905.



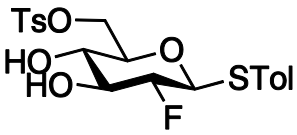
Tolyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-1-thio-β-D-glucopyranoside (13). A solution of compound **11** (5.2 g, 14.8 mmol) in dry DCM (10 mL) was cooled to 0 °C and HBr in AcOH (33wt%, 12.8 mL, 74 mmol) was added. The resulting solution was stirred at 4 °C overnight, after which the mixture was poured in ice water, diluted with EtOAc and washed with H_2O (2x) and sat. aq. NaCl. The combined aqueous layers were extracted with EtOAc and the resulting organic fractions were dried over Na_2SO_4, filtered, concentrated *in vacuo* and co-evaporated with toluene (3x). The crude product **12** was then used in the next reaction step without further purification. (TLC: R_f 0.42 (PE/EtOAc, 2/1, v/v); IR (neat, cm^{-1}): 729, 1038, 1209, 1367, 1744; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 6.55 (d, 1H, *J* = 4.3 Hz, H-1), 5.63 (dt, 1H, *J* = 9.4, 11.2 Hz, H-3), 5.12 (t, 1H, *J* = 9.9 Hz, H-4), 4.55 (ddd, 1H, *J* = 4.3, 9.4, 49.4 Hz, H-2), 4.29-4.37 (m, 2H, H-5, H-6), 4.10-4.15 (m, 1H, H-6), 2.09 (s, 3H, CH_3 Ac), 2.09 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 170.1, 169.5, 169.2 (C=O Ac), 86.1 (d, *J* = 197 Hz, C-2), 85.3 (d, *J* = 25 Hz, C-1), 71.9 (C-5), 70.8 (d, *J* = 19 Hz, C-3), 66.3 (d, *J* = 7 Hz, C-4), 60.6 (C-6), 20.4, 20.3 (CH_3 Ac)). The crude bromide (~14.8 mmol) was dissolved in dry $CHCl_3$ (150 mL), *p*-toluenethiol (2.76 g, 22.2 mmol) and TBAB (0.95 g, 2.96 mmol, dissolved in 20 mL H_2O) were

added and the resulting emulsion was cooled to 0 °C. Subsequently KOH (1.66 g, 29.6 mmol, dissolved in 20 mL H_2O) was added during 10 minutes and the resulting emulsion was vigorously stirred at room temperature overnight. Next the organic layer was separated, washed with sat. aq. NaCl, dried over Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 33% EtOAc in PE) yielded the title compound as a yellowish oil (Yield: 5.11 g, 12.3 mmol, 83% over two steps). TLC: R_f 0.42 (PE/EtOAc, 2/1, v/v); $[\alpha]_D^{20}$ +7.8° (*c* 1, DCM); IR (neat, cm^{-1}): 727, 908, 1030, 1217, 1744; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.40 (d, 2H, *J* = 8.1 Hz, CH_{arom}), 7.08 (d, 2H, *J* = 7.9 Hz, CH_{arom}), 5.26 (dt, 1H, *J* = 9.1, 14.1 Hz, H-3), 4.86 (t, 1H, *J* = 9.8 Hz, H-4), 4.60 (dd, 1H, *J* = 1.6, 9.7 Hz, H-1), 4.10-4.17 (m, 2.5H, H-2, H-6), 4.01 (t, 0.5H, *J* = 9.2 Hz, H-2), 3.68 (ddd, 1H, *J* = 3.1, 4.3, 10.1 Hz, H-5), 2.30 (s, 3H, CH_3 STol), 2.01 (s, 3H CH_3 Ac), 1.98 (s, 3H, CH_3 Ac), 1.96 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 170.2, 169.6, 169.3 (C=O Ac), 138.9 (C_q Tol-CH_3), 134.5, 129.5 (CH_{arom}), 125.9 (C_q STol), 86.6 (d, *J* = 190 Hz, C-2), 83.8 (d, *J* = 24 Hz, C-1), 75.4 (C-5), 73.6 (d, *J* = 20 Hz, C-3), 67.8 (d, *J* = 7 Hz, C-4), 61.7 (C-6), 21.0 (CH_3 STol), 20.5, 20.4, 20.3 (CH_3 Ac); HRMS: $[M+Na]^+$ calcd for $C_{19}H_{23}FO_7SNa$ 437.1041, found 437.1039.

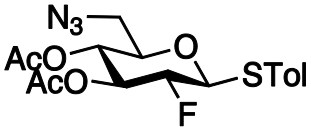


Tolyl 2-deoxy-2-fluoro-1-thio-β-D-glucopyranoside (14). A solution of compound **13** (2.72 g, 6.56 mmol) in dry MeOH (50 mL) was treated with NaOMe (1.06 g, 19.7 mmol) for 30 minutes at room temperature under an argon atmosphere. The mixture was neutralized with Amberlite-H^+, filtrated and concentrated *in vacuo* to yield the crude title compound as a white amorphous solid (Yield: quant.). TLC: R_f 0.46 (EtOAc); IR (neat, cm^{-1}): 766, 1009, 1047, 1364, 1614, 3277; ^{1}H NMR ($CDCl_3$/MeOH-d_4, 400 MHz, HH-COSY, HSQC): δ 7.45 (d, 2H, *J* = 8.0 Hz, CH_{arom}), 7.14 (d, 2H, *J* = 8.0 Hz, CH_{arom}), 4.64 (d, 1H, *J* = 9.6 Hz, H-1), 3.99 (dt, 1H, *J* = 9.2, 49.7 Hz, H-2), 3.87 (dd, 1H, *J* = 2.5, 12.2 Hz, H-6), 3.73 (dd, 1H, *J* = 4.7, 12.2 Hz, H-6), 3.63-3.70 (m, 1H, H-3), 3.32-3.39 (m, 2H, H-4, H-5), 2.35 (s, 3H, CH_3 STol); ^{13}C-APT NMR ($CDCl_3$/MeOH-d_4, 100 MHz, HSQC): δ 138.4 (C_q Tol-CH_3), 133.3, 129.5 (CH_{arom}), 127.2 (C_q STol),

89.5 (d, J = 186 Hz, C-2), 84.5 (d, J = 24 Hz, C-1), 79.9 (C-5), 75.9 (d, J = 18 Hz, C-3), 69.4 (d, J = 8 Hz, C-4), 61.4 (C-6), 20.7 (CH_3 STol); LC: R_t 5.53; linear gradient 10-90% B in 15 min; TLC-MS: m/z = 311.1 ($M+Na^+$).

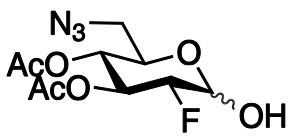


Tolyl 2-deoxy-2-fluoro-1-thio-6-*O*-(*p*-toluenesulfonyl)-β-D-glucopyranoside (15). Triol **14** (0.5 g, 1.74 mmol) was co-evaporated with dry dioxane (2x) and dissolved in dioxane (10 mL). The mixture was cooled to ~10 ºC, TEA (0.49 mL, 3.48 mmol) was added followed by the portion-wise addition of tosyl anhydride (0.62 g, 1.92 mmol). The reaction was stirred overnight at RT and subsequently diluted with EtOAc. The organic layer was washed with sat. aq. NaCl (3x), dried over Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 66% EtOAc in PE) furnished the title compound as a colored oil (Yield: 0.40 g, 0.90 mmol, 52%). TLC: R_f 0.71 (EtOAc); $[\alpha]_D^{20}$ -2.8º (*c* 1, DCM); IR (neat, cm^{-1}): 729, 1175, 1358, 2924, 3395; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.77 (d, 2H, J = 8.3 Hz, CH_{arom}), 7.32 (d, 2H, J = 8.1 Hz, CH_{arom}), 7.29 (d, 2H, J = 8.2 Hz, CH_{arom}), 7.03 (d, 2H, J = 8.1 Hz, CH_{arom}), 4.63 (bs, 2H, 3-OH, 4-OH), 4.50 (d, 1H, J = 9.5 Hz, H-1), 4.29 (d, 1H, J = 9.9 Hz, H-6), 4.21 (dd, 1H, J = 5.1, 11.0 Hz, H-6), 3.93 (dt, 1H, J = 9.1, 49.7 Hz, H-2), 3.70 (dt, 1H, J = 8.7, 15.4 Hz, H-3), 3.35-3.50 (m, 2H, H-4, H-5), 2.38 (s, 3H, CH_3 Ac), 2.29 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 145.0 (C_q Ts-CH_3), 138.4 (C_q Tol-CH_3), 133.5 (CH_{arom}), 132.3 (C_q STs), 129.8, 129.7, 129.6, 127.9 (CH_{arom}), 127.2 (C_q STol), 89.2 (d, J = 186 Hz, C-2), 84.1 (d, J = 24 Hz, C-1), 76.8 (C-5), 76.0 (d, J = 18 Hz, C-3), 69.1 (d, J = 7 Hz, C-4), 68.6 (C-6), 21.5, 21.0 (CH_3 STol, Ts); HRMS: $[M+Na]^+$ calcd for $C_{20}H_{23}FO_6S_2Na$ 465.0812, found 465.0811.



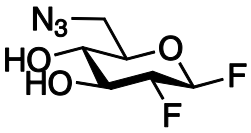
Tolyl 6-azido-2,6-di-deoxy-2-fluoro-1-thio-β-D-glucopyranoside (17). A solution of compound **15** (1.59 g, 3.6 mmol) and sodium azide (0.7 g, 10.8 mmol) in DMF (36 mL) was heated at 80 ºC overnight. The mixture was diluted with EtOAc,

washed with sat. aq. $NaHCO_3$ (2x) and H_2O (2x), dried over Na_2SO_4 and concentrated *in vacuo*. The crude azide **16** was used in the next step without further purification. (TLC: R_f 0.37 (PE/EtOAc, 1/1, v/v); IR (neat, cm^{-1}): 729, 1038, 1067, 1290, 2102, 3339; ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.46 (d, 2H, *J* = 8.1 Hz, CH_{arom}), 7.13 (d, 2H, *J* = 8.0 Hz, CH_{arom}), 4.54 (dd, 1H, *J* = 0.8, 9.6 Hz, H-1), 4.40 (bs, 1H, 3-OH), 4.17 (bs, 1H, 4-OH), 3.95 (dt, 1H, *J* = 9.1, 49.6 Hz, H-2), 3.66 (dt, 1H, *J* = 7.1, 14.6 Hz, H-3), 3.54 (d, 1H, *J* = 12.1 Hz, H-6), 3.37-3.41 (m, 2H, H-4, H-5), 3.34 (d, 1H, *J* = 13.3 Hz, H-6), 2.33 (s, 3H, CH_3 STol); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 139.1 (C_q Tol-CH_3), 134.7, 129.7 (CH_{arom}), 126.0 (C_q STol), 89.2 (d, *J* = 185 Hz, C-2), 84.1 (d, *J* = 24 Hz, C-1), 78.2 (C-5), 76.2 (d, *J* = 18 Hz, C-3), 69.7 (d, *J* = 7 Hz, C-4), 51.0 (C-6), 21.1 (CH_3 STol)). Crude azido compound **16** (~3.6 mmol) was treated with pyridine/Ac_2O (20 mL, 3/1, v/v) at RT overnight. The mixture was diluted with EtOAc, washed with sat. aq. NaCl (3x), dried over Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 25% EtOAc in PE) yielded the title compound as an amorphous solid (Yield: 0.98 g, 2.47 mmol, 69% over two steps). TLC: R_f 0.85 (PE/EtOAc, 1/1, v/v); $[\alpha]_D^{20}$ +37.8° (*c* 1, DCM); IR (neat, cm^{-1}): 729, 907, 1026, 1211, 1749, 2104; ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.48 (d, 2H, *J* = 8.0 Hz, CH_{arom}), 7.15 (d, 2H, *J* = 7.9 Hz, CH_{arom}), 5.31 (dt, 1H, *J* = 9.1, 14.1 Hz, H-3), 4.87 (t, 1H, *J* = 9.7 Hz, H-4), 4.66 (dd, 1H, *J* = 1.3, 9.7 Hz, H-1), 4.10 (dt, 1H, *J* = 9.3, 49.0 Hz, H-2), 3.68 (ddd, 1H, *J* = 2.6, 5.9, 9.7 Hz, H-5), 3.37 (dd, 1H, *J* = 2.5, 13.5 Hz, H-6), 3.26 (dd, 1H, *J* = 5.9, 13.5 Hz, H-6), 2.36 (s, 3H, CH_3 STol), 2.03 (s, 3H, CH_3 Ac), 2.00 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 169.6, 169.2 (C=O Ac), 139.3 (C_q Tol-CH_3), 135.1, 129.7 (CH_{arom}), 125.1 (C_q STol), 86.4 (d, *J* = 190 Hz, C-2), 83.7 (d, *J* = 24 Hz, C-1), 76.6 (C-5), 73.5 (d, *J* = 20 Hz, C-3), 68.7 (d, *J* = 7 Hz, C-4), 50.7 (C-6), 21.0 (CH_3 Stol), 20.4, 20.3 (CH_3 Ac); HRMS: $[M+Na]^+$ calcd for $C_{17}H_{23}FNO_5SNa$ 372.1275, found 372.1275.



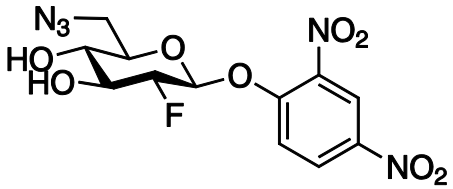
3,4-Di-*O*-acetyl-6-azido-2,6-di-deoxy-2-fluoro-α/β-D-glucopyranose (18). A solution of compound **17** (0.56 g, 1.41 mmol) in acetone/H_2O (16 mL, 3/1, v/v) was cooled to 0 °C followed by the addition of *N*-bromosuccinimide (0.75 g, 4.24

mmol). The resulting solution was stirred at +4 °C overnight, after which analysis by TLC showed complete conversion of the starting material into two lower-running products. The addition of extra *N*-bromosuccinimide (0.75 g, 4.24 mmol) and subsequent stirring at 0 °C for 3h resulted in full conversion into one spot as judged by TLC analysis. The reaction was quenched by the addition of sat. aq. $Na_2S_2O_3$, diluted with EtOAc and washed with sat. aq. NaCl (3x). The organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 33% EtOAc in PE) yielded the title compound as a colorless oil (Yield: 0.36 g, 1.22 mmol, 86%, α : β = 4 : 1). TLC: R_f 0.54 (PE/EtOAc, 1/1, v/v); IR (neat, cm^{-1}): 1024, 1213, 1747, 2104, 2924, 3443; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.56 (dt, 1H, *J* = 9.5, 12.0 Hz, H-3α), 5.48 (t, 1H, *J* = 3.0 Hz, H-1α), 5.30 (dt, 0.25H, *J* = 9.7, 14.0 Hz, H-3β), 4.97 (t, 1H, *J* = 9.8 Hz, H-4α), 4.88-4.93 (m, 0.25H, H-1β), 4.49 (ddd, 1H, *J* = 3.7, 9.6 49.5 Hz, H-2α), 4.34 (dd, 0.13H, *J* = 7.8, 9.1 Hz, H-2β), 4.15-4.26 (m, 1.13H, H-2β, H-5α), 4.71 (ddd, 0.25H, *J* = 3.7, 5.5, 13.6 Hz, H-5β), 3.37 (dd, 1H, *J* = 2.8, 13.4 Hz, H-6α), 3.33-3.35 (m, 0.5H, H-6β), 3.27 (dd, 1H, *J* = 5.8, 13.4 Hz, H-6α), 2.07 (s, 0.75H, CH_3 Ac-β), 2.06 (s, 3H, CH_3 Ac-α), 2.03 (s, 3H, CH_3 Ac-α), 2.03 (s, 0.75H, CH_3 Ac-β); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 170.5, 170.0 (C=O Ac), 94.4 (d, *J* = 23 Hz, C-1β), 90.3 (d, *J* = 189 Hz, C-2β), 89.9 (d, *J* = 26 Hz, C-1α), 87.6 (d, *J* = 192 Hz, C-2α), 72.9 (C-5β), 72.6 (d, *J* = 20 Hz, C-3β), 70.3 (d, *J* = 19 Hz, C-3α), 69.2 (d, *J* = 7 Hz, C-4β), 69.1 (d, *J* = 7 Hz, C-4α), 68.2 (C-5α), 50.7 (C-6), 20.7 (CH_3 Ac-α), 20.6 (CH_3 Ac-β), 20.5 (CH_3 Ac-α), 20.5 (CH_3 Ac-β); HRMS: $[M(amine)+H]^+$ calcd for $C_{10}H_{17}FNO_6$ 266.10344, found 266.10365.



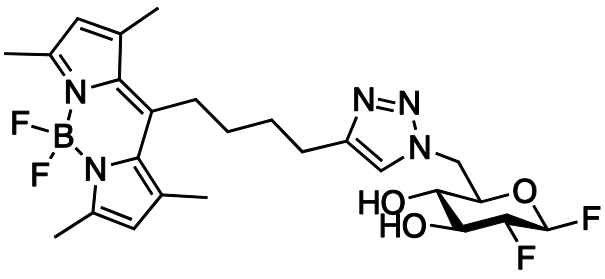
6-Azido-2,6-di-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (6). Compound **18** (60 mg, 0.21 mmol) was dissolved in dry DCM (4 mL) under an argon atmosphere. The solution was cooled to -45 °C and treated with DAST (63 μL, 0.52 mmol). The mixture was stirred at -45 °C for 1.5 h and quenched with MeOH (0.15 mL). After warming to RT the mixture was diluted with EtOAc, washed with sat. aq. NaCl, dried over Na_2SO_4 and concentrated *in vacuo* to yield the crude product as an anomeric mixture (α : β = 1 : 4). The anomers were partly

separated using flash column chromatography to yield the beta fluoride as a colorless oil (Yield: 39 mg, 0.13 mmol, 64%). TLC: R_f 0.50 (PE/EtOAc, 2/1, v/v); $[\alpha]_D^{20}$ +113.5º (*c* 1, DCM); IR (neat, cm^{-1}): 1028, 1099, 1207, 1749, 2104; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.48 (ddd, 1H, *J* = 3.8, 6.2, 52.0 Hz, H-1), 5.34 (dt, 1H, *J* = 8.3, 15.4 Hz, H-3), 5.10 (t, 1H, *J* = 9.3 Hz, H-4), 4.50 (dddd, 1H, *J* = 6.2, 8.0, 11.1, 49.9 Hz, H-2), 3.87 (ddd, 1H, *J* = 3.3, 5.8, 9.3 Hz, H-5), 3.46 (dd, 1H, *J* = 3.2, 13.5 Hz, H-6), 3.41 (dd, 1H, *J* = 5.8, 13.5 Hz, H-6), 2.10 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 169.7, 169.3 (C=O Ac), 105.7 (dd, *J* = 27, 218 Hz, C-1), 88.7 (dd, *J* = 28, 189 Hz, C-2), 73.0 (d, *J* = 4 Hz, C-5), 71.3 (dd, *J* = 9, 21 Hz, C-3), 68.0 (d, *J* = 7 Hz, C-4), 50.7 (C-6), 20.4, 20.4 (CH_3 Ac); HRMS: $[M+Na]^+$ calcd for $C_{10}H_{13}F_2N_3O_5Na$ 316.07155, found 316.07167. The peracetylated fluoride (85 mg, 0.29 mmol) was dissolved in MeOH (3 mL) and treated with cat. NaOMe (~ 1 mg) for 75 mins at RT. The mixture was neutralized with Amberlite-H^+, filtrated and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 66% EtOAc in PE) yielded compound **6** as a colorless oil (Yield: 62 mg, 0.29 mmol, 100%). TLC: R_f 0.13 (PE/EtOAc, 2/1, v/v); $[\alpha]_D^{20}$ +54.8º (*c* 1, MeOH); IR (neat, cm^{-1}): 1005, 1074, 1099, 2106, 3352; ^{1}H NMR (MeOH-d_4, 400 MHz, HH-COSY, HSQC): δ 5.39 (ddd, 1H, *J* = 3.5, 6.9, 53.2 Hz, H-1), 4.18 (dddd, 1H, *J* = 7.0, 8.9, 13.4, 51.6 Hz, H-2), 3.59-3.72 (m, 3H, H-3, H-5, H-6), 3.94 (dd, 1H, *J* = 5.1, 12.6 Hz, H-6), 3.43 (t, 1H, *J* = 9.3 Hz, H-4); ^{13}C-APT NMR (MeOH-d_4, 100 MHz, HSQC): δ 108.0 (dd, *J* = 26, 213 Hz, C-1), 93.5 (dd, *J* = 24, 185 Hz, C-2), 76.7 (d, *J* = 5 Hz, C-5), 75.1 (dd, *J* = 10, 18 Hz, C-3), 71.1 (d, *J* = 8 Hz, C-4), 52.2 (C-6); LC: R_t 7.26; linear gradient 10-90% B in 15 min; TLC-MS: *m*/*z* = 441.3 ($2M+Na^+$).



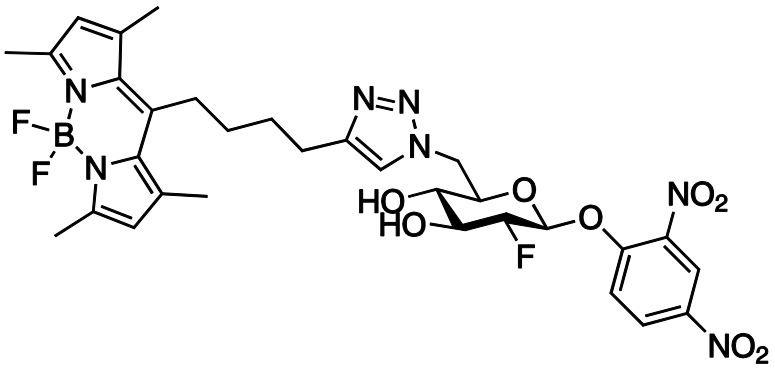
2,4-Di-nitrophenyl 6-azido-2,6-di-deoxy-2-fluoro-β-D-glucopyranoside (9). Compound **18** (58 mg, 0.20 mmol) was dissolved in dry DMF (3 mL). The mixture was cooled to 0 ºC and 2,4-dinitrofluorobenzene (56 mL, 0.44 mmol) and DABCO (91 mg, 0.81 mmol) were added. After 5h the mixture was diluted with EtOAc, washed with sat. aq. NaCl (3x), dried over Na_2SO_4 and

concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 66% EtOAc in PE) yielded the β-fused compound as a yellowish oil (Yield: 33 mg, 72 μmol, 36%). TLC: R_f 0.17 (PE/EtOAc, 2/1, v/v); $[\alpha]_D^{20}$ -88.9° (*c* 1, DCM); IR (neat, cm^{-1}): 1034, 1067, 1229, 1348, 1537, 1609, 1753, 2104; ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 8.77 (d, 1H, *J* = 2.7 Hz, CH_{arom}), 8.48 (dd, 1H, *J* = 2.8, 9.2 Hz, CH_{arom}), 7.45 (d, 1H, *J* = 9.2 Hz, CH_{arom}), 5.40-5.50 (m, 2H, H-1, H-3), 5.05 (t, 1H, *J* = 9.5 Hz, H-4), 4.72 (ddd, 1H, *J* = 7.2, 8.6, 49.8 Hz, H-2), 3.92 (ddd, 1H, *J* = 2.7, 7.5, 10.0 Hz, H-5), 3.47 (dd, 1H, *J* = 7.5, 13.5 Hz, H-6), 3.38 (dd, 1H, *J* = 2.7, 13.5 Hz, H-6), 2.13 (s, 3H, CH_3 Ac), 2.08 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 100 MHz, HSQC): δ 169.8, 169.4 (C=O Ac), 153.2, 142.3, 140.1 (C_q), 128.9, 121.7, 117.8 (CH_{arom}), 98.2 (d, *J* = 25 Hz, C-1), 88.4 (d, *J* = 192 Hz, C-2), 74.1 (C-5), 71.6 (d, *J* = 21 Hz, C-3), 68.5 (d, *J* = 7 Hz, C-4), 51.0 (C-6), 20.5, 20.5 (CH_3 Ac); TLC-MS: *m*/*z* = 480.1 ($M+Na^+$). A solution of peracetylated compound (33 mg, 72 μmol) in dry MeOH (1 mL) was treated with acetyl chloride (~4 drops) at RT until TLC analysis indicated complete conversion into one product (5 days). The mixture was quenched with TEA till pH ~ neutral, diluted with EtOAc and concentrated *in vacuo*. Purification using flash column chromatography (silica gel, 75% EtOAc in PE) furnished the title compound as a colorless oil (Yield: 24 mg, 64.2 μmol, 89%). TLC: R_f 0.25 (PE/EtOAc, 1/2, v/v); $[\alpha]_D^{20}$ -148.0° (*c* 0.5, MeOH); IR (neat, cm^{-1}): 1069, 1281, 1348, 1535, 1609, 2104, 3395; ^{1}H NMR (MeOH-d_4, 400 MHz, HH-COSY, HSQC): δ 8.74 (d, 1H, *J* = 2.8 Hz, CH_{arom}), 8.50 (dd, 1H, *J* = 2.8, 9.3 Hz, CH_{arom}), 7.66 (d, 1H, *J* = 9.3 Hz, CH_{arom}), 5.64 (dd, 1H, *J* = 3.2, 7.5 Hz, H-1), 4.35 (ddd, 1H, *J* = 7.6, 8.9, 51.3 Hz, H-2), 3.71-3.82 (m, 2H, H-3, H-5), 3.60 (dd, 1H, *J* = 2.3, 13.4 Hz, H-6), 3.49 (dd, 1H, *J* = 7.0, 13.4 Hz, H-6), 3.42 (t, 1H, *J* = 9.4 Hz, H-4); ^{13}C-APT NMR (MeOH-d_4, 100 MHz, HSQC): δ 154.8, 143.2, 141.2 (Cq), 129.8, 122.2, 118.9 (CH_{arom}), 99.2 (d, *J* = 25 Hz, C-1), 92.8 (d, *J* = 187 Hz, C-2), 77.5 (C-5), 75.7 (d, *J* = 17 Hz, C-3), 71.5 (d, *J* = 8 Hz, C-4), 52.5 (C-6); TLC-MS: *m*/*z* = 764.6 ($2M+NH_4^+$).



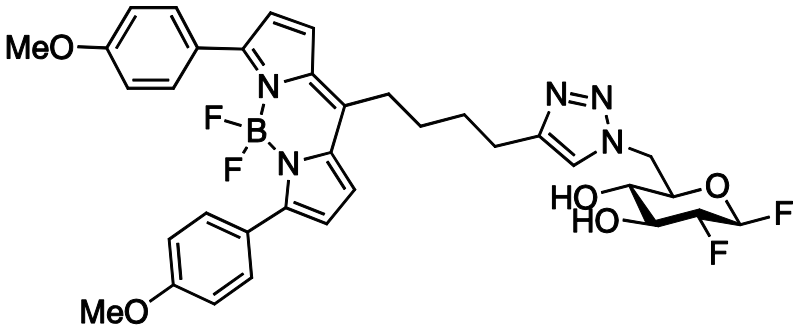
MW448 (4). Compound **6** (5.8 mg, 27.7 μmol) and Bodipy-alkyne **10** (9.98 mg, 30.4 μmol) were together dissolved in DMF (0.5 mL),

followed by the addition of sodium ascorbate (4.1 μL, 1 M) and copper(II)sulfate (2.7 μL, 1 M). The solution was stirred at 45 °C overnight and extra sodium ascorbate and copper(II)sulfate were added. After 2 days, the mixture was concentrated *in vacuo* and purified using flash column chromatography (silica gel, 5% MeOH in DCM) to yield the title compound as a bright orange solid (Yield: 8.4 mg, 15.6 μmol, 56%). TLC: R_f 0.23 (DCM/MeOH, 9/1, v/v); IR (neat, cm^{-1}): 986, 1202, 1510, 1551, 2926, 3333; ^1H NMR ($CDCl_3$/MeOH-d_4, 600 MHz, HH-COSY, HSQC): δ 6.06 (s, 2H, CH-pyrole), 5.26 (ddd, 1H, *J* = 3.6, 6.2, 52.6 Hz, H-1), 4.77 (d, 1H, *J* = 14.1 Hz, H-6), 4.65 (dd, 1H, *J* = 4.2, 13.9 Hz, H-6), 4.15 (dddd, 1H, *J* = 7.9, 8.1, 12.9, 51.6 Hz, H-2), 3.67-3.78 (m, 2H, H-3, H-5), 3.16 (t, 1H, *J* = 9.1 Hz, H-4), 3.01 (bs, 2H, CH_2), 2.80 (bs, 2H, CH_2), 2.50 (s, 6H, CH_3), 2.40 (s, 6H, CH_3), 1.93 (bs, 2H, CH_2), 1.71 (bs, 2H, CH_2); ^{13}C-APT NMR ($CDCl_3$, 125 MHz, HSQC): δ 153.6, 145.8, 140.3, 131.1 (C_q), 121.5 (CH_{arom}), 106.2 (dd, *J* = 22, 180 Hz, C-1), 91.4 (dd, *J* = 20, 155 Hz, C-2), 74.1 (d, *J* = 4 Hz, C-5), 73.3 (dd, *J* = 8, 15 Hz, C-3), 69.4 (d, *J* = 7 Hz, C-4), 50.7 (C-6), 31.1, 29.4, 29.2, 27.8, 25.0 (CH_2), 16.0, 14.0 (CH_3); LC/MS: R_t 8.23; linear gradient 10-90% B in 15 min; ESI-MS: *m/z* = 537.7 ($M+H^+$); HRMS: $[M+H]^+$ calcd for $C_{25}H_{33}BF_4N_5O_3$ 538.26071, found 538.26041.



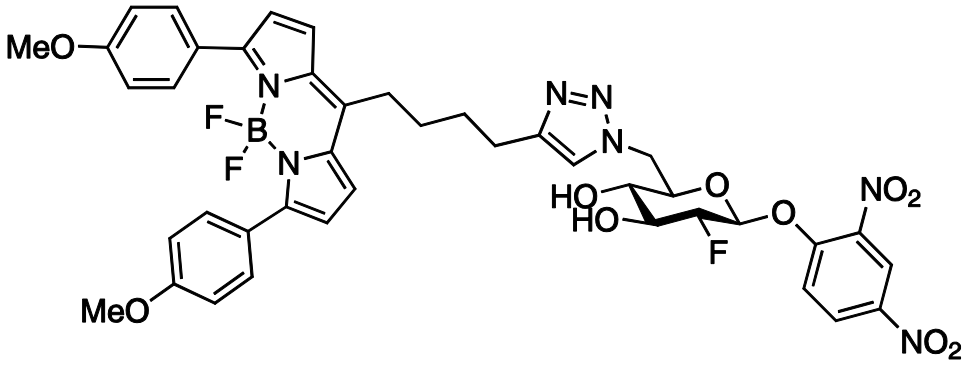
MW454 (7). Compound **9** (9.2 mg, 24.6 μmol) and Bodipy-alkyne **10** (10.2 mg, 31.1 μmol) were together dissolved in DMF (0.5 mL), followed by the addition of sodium ascorbate (3.7 μL, 1 M) and copper(II)sulfate (2.5 μL, 1 M). The solution was stirred at 45 °C overnight and extra sodium ascorbate and copper(II)sulfate were added. After 2 days, the mixture was concentrated *in vacuo* and purified using flash column chromatography (silica gel, 5% MeOH in DCM) to yield the title compound as an orange solid (Yield: 3.6 mg, 5.1 μmol, 21%). TLC: R_f 0.18 (CH_2Cl_2/MeOH, 19/1, v/v); IR (neat, cm^{-1}): 1070, 1200, 1348, 1541, 1609, 2102, 3350; ^1H NMR ($CDCl_3$/MeOH-d_4, 600

MHz, HH-COSY, HSQC): δ 8.69 (d, 1H, *J* = 2.6 Hz, CH_{arom}), 8.27 (dd, 1H, *J* = 2.6, 9.1 Hz, CH_{arom}), 7.33 (s, 1H, CH-triazole), 6.96 (d, 1H, *J* = 9.2 Hz, CH_{arom}), 6.05 (s, 1H, CH-pyrole), 5.21 (dd, 1H, *J* = 2.9, 7.5 Hz, H-1), 4.83 (dd, 1H, *J* = 1.3, 14.3 Hz, H-6), 4.43-4.50 (m, 1.5 H, H-2, H-6), 4.37 (t, 0.5H, *J* = 8.2 Hz, H-2), 3.99 (t, 1H, *J* = 7.8 Hz, H-5), 3.81 (dt, 1H, *J* = 8.9, 15.8 Hz, H-3), 3.33 (t, 1H, *J* = 9.4 Hz, H-4), 2.93-3.02 (m, 2H, CH_2), 2.74-2.79 (m, 2H, CH_2), 2.49 (s, 6H, CH_3), 2.37 (bs, 6H, CH_3), 1.81-1.94 (m, 2H, CH_2), 1.58-1.70 (m, 2H, CH_2); ^{13}C-APT NMR ($CDCl_3$/MeOH-d_4, 125 MHz, HSQC): δ 153.4, 145.7, 141.9, 131.2 (C_q), 128.6, 121.3, 117.9 (CH_{arom}), 98.6 (d, *J* = 21 Hz, C-1), 90.7 (d, *J* = 157 Hz, C-2), 74.7 (C-5), 74.4 (d, *J* = 15 Hz, C-3), 70.2 (d, *J* = 7 Hz, C-4), 50.6 (C-6), 31.2, 29.4, 27.9, 25.1 (CH_2), 16.2, 14.2 (CH_3); LC/MS: R_t 8.94; linear gradient 10-90% B in 15 min; ESI-MS: *m*/*z* = 701.9 ($M+H^+$); HRMS: $[M+H]^+$ calcd for $C_{31}H_{36}BF_3N_7O_8$ 702.26650, found 702.26640.



MW461 (5). Compound **6** (7.6 mg, 36.3 μmol) and Bodipy-alkyne **10** (24.4 mg, 50.3 μmol) were together dissolved in DMF (0.5 mL), followed by the addition of sodium ascorbate (5.4 μL, 1 M and copper(II)sulfate (3.6 μL, 1 M). The solution was stirred at 45 °C overnight and extra sodium ascorbate and copper(II)sulfate were added. Then the mixture was concentrated *in vacuo* and purified using HPLC to yield the title compound as a dark blue solid (Yield: 9.2 mg, 13.2 μmol, 36%). IR (neat, cm^{-1}): 1067, 1142, 1468, 1566, 2853, 2920, 3366; ^{1}H NMR ($CDCl_3$/MeOH-d_4, 600 MHz, HH-COSY, HSQC): δ 7.83 (d, 4H, *J* = 8.8 Hz, CH_{arom}), 7.47 (s, 1H, CH-pyrole), 7.27 (d, 2H, *J* = 4.3 Hz, CH-pyrole), 6.94 (d, 4H, *J* = 8.8 Hz, CH_{arom}), 6.61 (d, 2H, *J* = 4.2 Hz, CH-pyrole), 5.27 (ddd, 1H, *J* = 3.8, 6.5, 52.6 Hz, H-1), 4.75 (dd, 1H, *J* = 2.3, 14.6 Hz, H-6), 4.61 (dd, 1H, *J* = 5.9, 14.6 Hz, H-6), 4.16 (dddd, 1H, *J* = 7.0, 8.5, 12.8, 51.1 Hz, H-2), 3.85 (s, 6H, OMe), 3.66-3.77 (m, 2H, H-3, H-5), 3.17 (t, 1H, *J* = 9.4 Hz, H-4), 2.99 (app t, 2H, *J* = 7.1 Hz, CH_2), 2.79 (t, 2H, *J* = 6.5 Hz, CH_2), 1.88 (bs, 4H, CH_2); ^{13}C-APT NMR ($CDCl_3$/MeOH-d_4, 125 MHz, HSQC): δ 160.4,

157.5, 147.4, 144.6, 136.1 (C_q), 130.9, 126.7 (CH_{arom}), 125.1 (C_q), 123.1, 119.9, 113.6 (CH_{arom}), 106.4 (dd, *J* = 23, 180 Hz, C-1), 91.4 (dd, *J* = 21, 154 Hz, C-2), 74.3 (d, *J* = 4 Hz, C-5), 73.5 (dd, *J* = 8, 15 Hz, C-3), 69.3 (d, *J* = 7 Hz, C-4), 55.2 (OMe), 50.1 (C-6), 33.0, 30.3, 29.6, 25.0 (CH_2); LC/MS: R_t 9.30; linear gradient 10-90% B in 15 min; ESI-MS: *m/z* = 693.9 ($M+H^+$). HRMS: $[M+H]^+$ calcd for $C_{35}H_{37}BF_4N_5O_5$ 694.28245, found 694.28199.



MW462 (8). Compound **9** (13.5 mg, 36.0 μmol) and Bodipy-alkyne **10** (35.0 mg, 72 μmol) were together dissolved in DMF (1 mL), followed by the addition of sodium ascorbate (5.4 μL, 1 M) and copper(II)sulfate (3.6 μL, 1 M). The solution was stirred at 45 °C overnight and extra sodium ascorbate and copper(II)sulfate were added. After 2 days, the mixture was concentrated *in vacuo* and purified using HPLC to yield the title compound as a blue solid (Yield: 10 mg, 11.6 μmol, 32%). IR (neat, cm^{-1}): 1069, 1142, 1466, 1572, 1684, 2853, 2926, 3395; ^1H NMR ($CDCl_3$/MeOH-d_4, 600 MHz, HH-COSY, HSQC): δ 8.69 (d, 1H, *J* = 2.7 Hz, CH_{arom}), 8.30 (dd, 1H, *J* = 2.8, 9.2 Hz, CH_{arom}), 7.81 (d, 4H, *J* = 8.8 Hz, CH_{arom}), 7.30 (s, 2H, CH-pyrole), 7.25 (d, 2H, *J* = 4.3 Hz, CH-pyrole), 7.00 (d, 1H, *J* = 9.3 Hz, CH_{arom}), 6.94 (d, 4H, *J* = 8.9 Hz, CH_{arom}), 6.60 (d, 2H, *J* = 4.3 Hz, CH-pyrole), 5.26 (dd, 1H, *J* = 3.0, 7.6 Hz, H-1), 4.80 (dd, 1H, *J* = 2.3, 14.5 Hz, H-6), 4.46 (app dd, 1H, *J* = 7.9, 14.6 Hz, H-6), 4.39 (ddd, 1H, *J* = 7.8, 8.7, 51.0 Hz, H-2), 3.99-4.03 (m, 1H, H-5), 3.77-3.86 (m, 1H, H-3), 3.85 (s, 6H, OMe), 3.30 (t, 1H, *J* = 9.4 Hz, H-4), 2.97 (t, 2H, *J* = 7.2 Hz, CH_2), 2.67-2.80 (m, 2H, CH_2), 1.80-1.90 (m, 4H, CH_2); ^{13}C-APT NMR ($CDCl_3$/MeOH-d_4, 125 MHz, HSQC): δ 160.4, 157.5, 153.4, 144.5, 141.8, 139.9, 136.0 (C_q),130.8, 128.6, 126.7 (CH_{arom}), 125.0 (C_q), 123.0, 121.3, 119.9, 117.8, 113.6 (CH_{arom}), 98.4 (d, *J* = 21 Hz, C-1), 90.7 (d, *J* = 157 Hz, C-2), 74.6 (C-5), 74.2 (d, *J* = 15 Hz, C-3), 70.2

(d, J = 7 Hz, C-4), 55.2 (OMe), 50.5 (C-6), 32.9, 31.8, 29.2, 25.0 (CH_2); LC/MS: R_t 9.90; linear gradient 10-90% B in 15 min; ESI-MS: m/z = 857.9 ($M+H^+$). HRMS: $[M+H]^+$ calcd for $C_{41}H_{40}BF_3N_7O_{10}$ 858.28834, found 858.28884.

Two-step labeling: Staudinger ligation versus copper catalyzed click-reaction

Recombinant purified glucocerebrosidase (5 μL, 1 mg/mL) was added to PBS (5 μL, pH 7.4) containing BSA (5 mg/mL) and KY170 **3** (200 μM). After incubating on ice for 30 min, 5 μL of the solution was added to 45 μL McIlvain buffer (50 mM citric acid, 100 mM Na_2HPO_4, pH 5.2 containing 0.2% sodium taurocholate, 0.1% Triton X-100) and the resulting mixture was incubated at 37°C for 30 minutes. The inhibited enzyme was aliquoted over eppendorf tubes (5 μL per eppendorf). *Staudinger ligation:* To remove excess probe, the proteins were precipitated using chloroform/methanol precipitation.[5] The protein pellet was redissolved in McIlvain buffer (10 μL) containing 6M Urea and 6 mM beta-mercaptoethanol. To this was added 1 μL of Staudinger-biotin phosphane **20** (4 mM stock solution in DMF stored under inert atmosphere at -80°C) and the resulting mixture was incubated at 37°C for 2 hours. The reaction was quenched by adding Laemli sample buffer (4×) and boiling for 5 min.

Click reaction: To remove excess probe, the proteins were precipitated using chloroform/methanol precipitation. The protein pellet was redissolved in 100 μL NaOAc buffer (50 mM, pH 6.0) containing SDS (1%). $CuSO_4$ (1 μL, 0.1 M), dithiothreitol (0.5 μL, 0.1 M) and either biotin-alkyne **21** or BODIPY-alkyne **10** (1 μL, 200 μM) were added and the reaction was incubated 1h at room temperature. The proteins were precipitated by the addition of ice-cold acetone (1 mL) and incubating at -20°C for 1h before being pelletized by centrifugation at 16,000× g at 4°C for 20 min. The supernatant was removed and the protein pellet was redissolved in Laemli sample buffer (2×).

The proteins were denatured by boiling for 5 min before being loaded on a 7.5% SDS-PAGE gel. Proteins modified with biotin as a reporter group were transferred to a PVDF-membrane. The membranes were blocked with 0.5% bovine serum albumin in TBS-TWEEN (0.1% TWEEN-20) for

30 min and incubated with Streptavidine-HRP (Amersham Bioscience 1:5000) for 30 min at ambient temperature. The membranes were briefly washed with TBS containing 0.1% TWEEN-20 and TBS followed by visualisation of the biotinylated proteins with an ECL+ Kit (Amersham Bioscience).

Optimization of the click reaction

Optimization of the time of labeling: To GBA-1 (100 ng) pre-treated with KY170 **3** (1 μL, 100 μM) at 37°C for 30 min was added NaOAc buffer (80 μL, 50 mM pH 6.0, 0.1% SDS). A fresh mixture of TBTA (10 μL, 2 mM in DMF), $CuSO_4$ (1 μL, 0.1 M in H_2O), DTT (0.5 μL, 0.1 M in H_2O) and BODIPY-alkyne **10** (0.5 μL, 2 mM in MeCN) was prepared, added to the enzyme solution and the resulting mixture was incubated for respectively 1, 2, 4 or 16 hours at room temperature. The proteins were precipitated by the addition of ice-cold acetone (1 mL) followed by incubating at -20°C for 20 min and centrifugation (16,000× g, 15 min) at 4°C. The proteins were redissolved in 2× sample buffer (15 μL) and loaded on a 7.5% SDS-PAGE gel. The fluorescence was measured in the wet gel slabs using the CY2 settings (λ_{ex} 488, λ_{em} 520) on a Typhoon Variable Mode Imager (Amersham Biosciences).

Optimization of the reductor: To GBA-1 (100 ng) pre-treated with KY170 **3** (1 μL, 100 μM) at 37°C for 30 min was added NaOAc buffer (80 μL, 50 mM pH 6.0, 0.1% SDS). To a fresh mixture of TBTA (10 μL, 2 mM in DMF), copper (II) sulfate (1 μL, 0.1 M in H_2O) and BODIPY-alkyne **10** (0.5 μL, 2 mM in MeCN) was added respectively DTT (0.5 μL, 0.1 M in H_2O), TCEP (0.5 μL, 0.2 M in H_2O) or sodium ascorbate (0.5 μL, 0.2 M in H_2O) before being added to the enzyme solution. The resulting mixture was incubated for 16 hours at room temperature. The reaction was quenched and the labeled proteins were visualized as described above.

Optimization of amount of copper (II) sulfate: To GBA-1 (100 ng) pre-treated with KY170 **3** (1 μL, 100 μM) at 37°C for 30 min was added NaOAc buffer (80 μL, 50 mM pH 6.0, 0.1% SDS). A fresh mixture of TBTA (10 μL, 2 mM in DMF), copper (II) sulfate (1 μL, 100× stock in H_2O, final concentrations: 50, 100, 500 and 1000 μM), DTT (0.5 μL, 100× stock in H_2O, 0.5 equivalents

compared to copper (II) sulfate) and BODIPY-alkyne **10** (0.5 μL, 2 mM in MeCN) was prepared, added to the enzyme solution and the resulting mixture was incubated for 16 hours at room temperature. The reaction was quenched and the labeled proteins were visualized as described above.

Optimization of the amount of SDS: To GBA-1 (100 ng) pre-treated with KY170 **3** (1 μL, 100 μM) at 37°C for 30 min was added NaOAc buffer (80 μL, 50 mM pH 6.0) containing SDS (respectively 0.05%, 0.1%, 0.5% or 1% (w/v)). A fresh mixture of TBTA (10 μL, 2 mM in DMF), copper (II) sulfate (1 μL, 0.1 M in H_2O), DTT (0.5 μL, 0.1 M in H_2O) and BODIPY-alkyne **10** (0.5 μL, 2 mM in MeCN) was prepared, added to the enzyme solution and the resulting mixture was incubated for 16 hours at room temperature. The reaction was quenched and the labeled proteins were visualized as described above.

*Optimization of the amount of BODIPY-alkyne **10**:* To GBA-1 (100 ng) pre-treated with KY170 **3** (1 μL, 100 μM) at 37°C for 30 min was added NaOAc buffer (80 μL, 50 mM pH 6.0, 0.1% SDS). A fresh mixture of TBTA (10 μL, 2 mM in DMF), copper (II) sulfate (1 μL, 0.1 M in H_2O), DTT (0.5 μL, 0.1 M in H_2O) and BODIPY-alkyne **10** (0.5 μL, 100× stock in MeCN, final concentrations: 0.1, 0.5, 1, 5 and 10 μM) was prepared, added to the enzyme solution and the resulting mixture was incubated for 16 hours at room temperature. The reaction was quenched and the labeled proteins were visualized as described above.

Competition experiments

Protocol for irreversible inhibitors: 9 μL of GBA-1 (20 ng) in reaction buffer was preincubated with CBE (1 μL, 2 mM final concentration) at 37°C for 30 min followed by incubation with the probe (**3**: 10 μM for 30 min, **4**: 400 μM for 6 h and **5**: 100 μM for 6 h). The reaction was quenched either by the addition of 4× SDS-PAGE sample buffer (5 μL) (**4** and **5**) or by the addition of the click buffer (**3**). The samples were treated and analyzed by SDS-PAGE as described above for the labeling experiments.

Protocol for reversible inhibitors: 9 μL of GBA-1 (20 ng) in reaction buffer was incubated with AMP-DNM (1 μL, 2 mM final concentration) in combination with 1 μL of the probe (final concentrations: **3**: 10 μM for 30 min, **4**: 400 μM for 6 h and **5**: 100 μM for 6 h). After quenching of the reaction, the samples were handled as described above.

Protocol for heat-inactivation: 1 μL SDS (10%) was added to 9 μL of GBA-1 (20 ng) in reaction buffer. The enzyme was heated at 100°C for 5 min, cooled to rt after which 1 μL of the probe (final concentrations: **3**: 10 μM for 30 min, **4**: 400 μM for 6 h and **5**: 400 μM for 6 h) was added. After incubating the sample at 37°C for 30 min, the samples were handled as described above.

References and footnotes.

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[5] Click protocol adapted from: F. Kaschani, S. H. L. Verhelst, P. F. van Swieten, M. Verdoes, C. S. Wong, Z. Wang, M. Kaiser, H. S. Overkleeft, M. Bogyo, R. A. L. van der Hoorn, *Plant J.* **2009**, *57*, 373.

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